Gill cells – an alternative to whole fish for toxicity tests

In order to protect human health and the environment, chemicals have to undergo risk assessment before they come onto the market. For this purpose, thousands of animal tests are carried out every year. Eawag researchers have now shown that the acute toxicity of chemicals to fish can also be reliably predicted with a rainbow trout gill cell line. An international round robin test marks the next step on the long road to certification of the new assay.

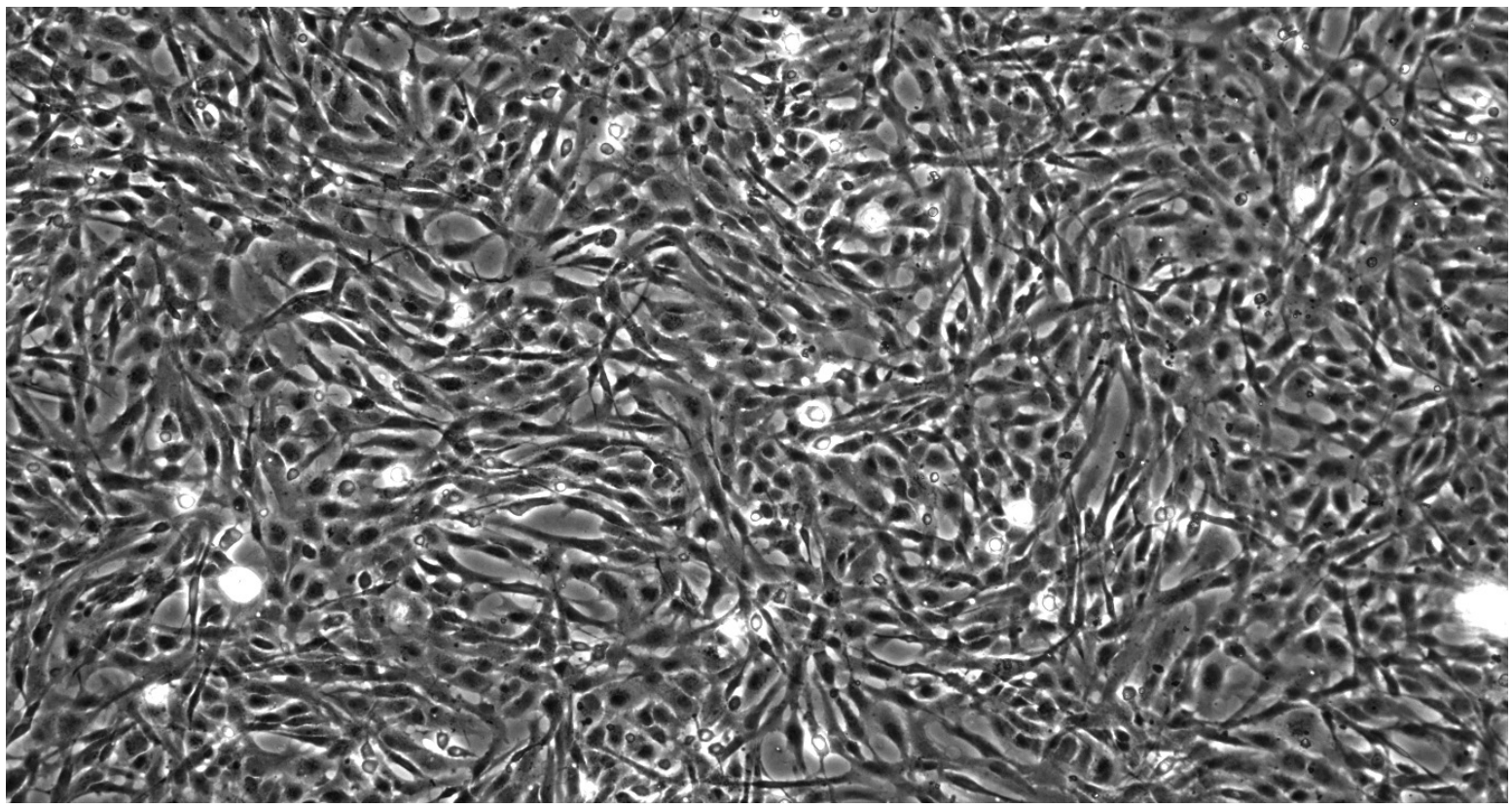

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Fig. 1: The gill cell line derived from rainbow trout (RTgill-W1) is suitable for chemical toxicity testing.

Although chemical substances are indispensable in our daily lives, they may pose risks to human health and the environment. Accordingly, the approval procedure involves an assessment of their physico-chemical and toxicological properties. Under the EU Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (Reach), all industrial chemicals placed on the market in quantities of more than one tonne per year are required to undergo toxicity testing. Such tests also form part of the approval procedure for pesticides, biocides and pharmaceuticals.

The Reach legislation covers not only all new chemicals but also existing substances which have not been adequately assessed to date. While this certainly represents a major step to-

wards safer management of chemicals, the testing required – particularly for toxicological assessment – is time-consuming and labour-intensive and, most critically, involves the use of large numbers of animals. In Switzerland alone, according to the Federal Veterinary Office (http://tv-statistik.ch/de/statistik/index.php), around 18,500 fish were used in tests for the protection of human/animal health and the environment in 2011; in 2012, the figure was just under 5,000. Toxicity tests involving animals are also carried out in other areas such as basic research or product development. Estimates suggest that, using currently accepted methods, it would simply not be possible to test all the chemicals for which safety evaluations are required under Reach [1].

First step: identifying candidate substances

There is thus an urgent need for novel testing strategies. The aim should be to develop methods permitting effective prioritization of chemicals, so that candidate substances can be identified for which animal testing is then actually needed. A combination of different methods would appear to be the most promising approach. For example, mathematical simulations could be combined with biological experimental models: with the former, the properties of chemicals can be used to predict their biological effects or distribution in the tissues of an organism, while the latter allow conclusions to be drawn concerning toxicologically relevant effects. Suitable for this purpose are test systems based on enzymes or cells, which require limited laboratory space, generate less toxic waste and permit automated high-throughput screening.

However, methods involving small or early life-stage organisms can also be useful. The new Test Guideline 236 of the Organization for Economic Cooperation and Development (OECD), which came into force in autumn 2013, allows the acute toxicity of chemicals to be determined in zebrafish embryos, rather than in juvenile or adult fish. As shown by numerous studies including research by Eawag scientists, embryos respond just as sensitively to short-term exposure to high concentrations of chemicals (i.e. acute exposure) as fish at later stages of development [2, 3, video]. Although the embryo test takes four days – like the traditional fish acute toxicity test (OECD Test Guideline 203) – its format is much smaller and more flexible. In future, therefore, regulatory submissions for chemicals from industry can increasingly be expected to include data from the embryo test instead of the traditional fish

Did you know that:

- fish are the most frequently used vertebrates in ecotoxicology testing?
- fish are used to assess the risks of chemicals and industrial effluents?
- the acute toxicity test is the most commonly used test involving fish?

toxicity test. In addition, under current regulations, the method involving zebrafish embryos is not considered to be an animal test and is ethically less problematic [4]. The embryo test also makes it possible to detect complex effects, e.g. on organ development or on behaviour, and can be used in high-throughput procedures. For this reason, it has even been proposed to be explored as an alternative to fish tests lasting several weeks [5].

Do cell lines offer an alternative to adult fish?

We investigated the question whether acute toxicity could not also be predicted using fish cells. The underlying idea was as follows: The fish acute toxicity test is used, as mentioned above, to determine lethality in fish exposed to high concentrations of chemicals over a four-day period. Rapid and massive exposure of this kind is associated in most cases with severe damage to cells and tissues which are in direct contact with the water. Particularly exposed – because of their large surface area – are the gills.

Damage to gill cells impairs vital functions such as oxygen delivery and ion exchange. This suggests that, if the impairment of viability can be determined in cell cultures, it should be possible to predict the chemical concentrations lethal to fish.

Based on these considerations, we decided to use a cell line derived from the gills of a rainbow trout *(Oncorhynchus mykiss)*. The cell line, established at the University of Waterloo in Canada, is known as Rainbow Trout gill – Waterloo 1 (RTgill-W1) [6] (Fig. 1). As this is a permanent or "immortalized" cell line, it can be reproduced and cultured indefinitely. It thus potentially offers a real alternative to animal tests.

Comparable results obtained with RTgill-W1 cells

A detailed account of how a cell line can be derived, and of the initial challenges we faced in establishing a test protocol with the RTgill-W1 cell line, was given in *Eawag News 68* (February 2010). The key elements in the development of an effective protocol were: (1) the use of a minimal medium not containing any components which provide additional protection against chemicals; (2) a dosing method permitting uniform exposure of cells to test chemicals; and (3) determination of the concentrations of chemicals actually present in the exposure medium [7]. The exposure conditions of the RTgill-W1 cells were thus made to conform as closely as possible to those experienced by gill cells in the fish acute toxicity test.

In order to determine – analogously to the fish acute toxicity test – the concentrations leading to a 50 per cent reduction in cell viability among the cell population (EC_{50}), we generated concentration-response curves for a total of 35 organic chemicals [8]. The selected chemicals differed primarily in their toxic modes of action (e.g. non-specific baseline toxicity, reactivity, neurotoxicity), in their physico-chemical properties (e.g. volatility and hydrophobicity) and in their toxicity to fish (low to high). This means that they are representative of many other substances.

The EC_{50} values correlated very well with the LC_{50} values (concentrations lethal to 50 per cent of test organisms) from the fish acute toxicity test (Fig. 2). The differences between the effective and lethal concentrations were found to be less than 5-fold for up to 73 per cent of the chemicals. This was true for substances with a broad spectrum of modes of action, a variety of physico-chemical properties, and toxicities ranging from low to high. For example, the reactive chemicals tested, such as dimethylbutadiene and hexachlorophene, differ over 3 orders of magnitude in their hydrophobicity and toxicity. Even so, the cell test was equally powerful in predicting their acute toxicity to fish. Only for five substances was the difference between the EC_{50} and LC_{50} values more than 10-fold; here, the cell test appeared to be less sensitive than the fish test. Three of these chemicals – the insecticides permethrin and lindane and the alkaloid caffeine – have neurotoxic effects and bind specifically to ion channels in the nervous system which are not present in the gill cell line.

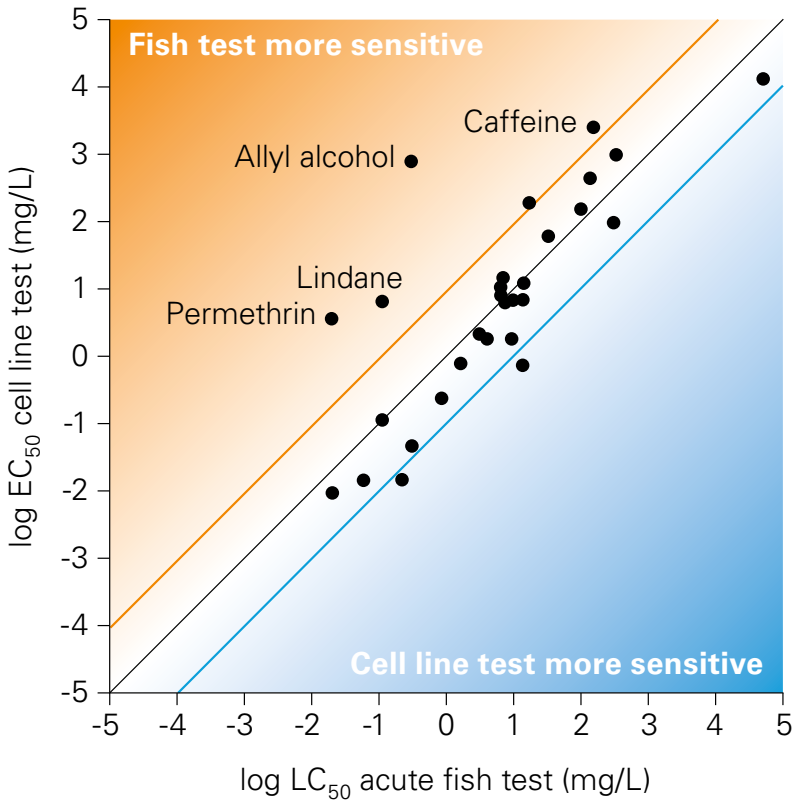

Fig. 2: Comparison of effect concentrations observed in the acute fish test (LC_{50}) and the cell line test (EC_{50}). The black line indicates identical effect concentrations for the two test systems, and the coloured lines indicate a 10-fold deviation from this line.

The most striking outlier was allyl alcohol: here, a response was only observed in the cell test with far higher concentrations than in the fish test. It is known from studies in mammals that this substance is transformed by the enzyme alcohol dehydrogenase into acrolein, a potent toxicant. Acrolein also showed highly toxic effects in RTgill-W1 cells. This suggests that the enzymatic transformation of allyl alcohol into acrolein is absent or incomplete in RTgill-W1 cells; similar results were also obtained in the above-mentioned test based on zebrafish embryos [3].

The long road to certification

The same chemicals selected for the RTgill-W1 cell line assay were also studied using the embryo test [3]. This allowed us to compare the two methods. Here, too, an excellent correlation was found between the toxicity values for the two tests. For almost all effect concentrations, a less than 10-fold difference was observed (Fig. 3). This was also true of allyl alcohol. The

greatest difference was seen in the case of rotenone, which is known to be highly toxic to fish. In the cell test, it was found to be 780-fold more toxic than in the embryo test. The results demonstrate that the RTgill-W1 cell line assay has a potential similar to that of the zebrafish embryo to serve as an alternative to the traditional fish acute toxicity test. A test system of this kind no longer requires the use of any experimental animals.

But there is still a long way to go before the cell line test is internationally accepted and can be widely adopted. First, it must be determined whether the method can also be established with reproducibly comparable results by industrial and other research laboratories. To this end, we recently initiated an international round robin test. If the test protocol is shown to be robust, we aim to secure OECD certification, as was obtained for the method based on zebrafish embryos. However, the certification process takes a long time, as it involves harmonization of the proposals and views of experts from many different member countries. In the case of the embryo test, the time elapsing from submission of the original test protocol to approval was seven years – a relatively short period.

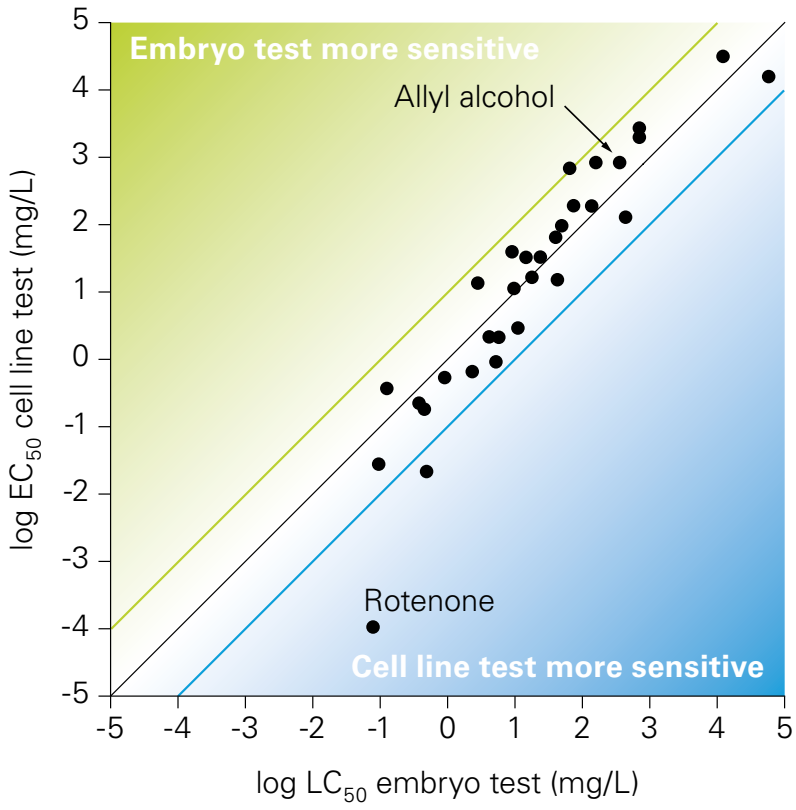

Fig. 3: Comparison of effect concentrations observed in the embryo test (LC_{50}) and the cell line test (EC_{50}). The black line indicates identical effect concentrations for the two test systems, and the coloured lines indicate a 10-fold deviation from this line.

Certification by organizations with a global reach is very important, as it is the only way of ensuring that test results are internationally recognized. The OECD and other organizations involved, such as the European Union Reference Laboratory for alternatives to animal testing are endeavouring to speed up the certification process. This is urgently needed to facilitate access to new methods for the assessment of chemicals. In addition, we are already making the cell line test protocol available on request since – aside from chemical assessment – it could also offer major benefits for product development or for the testing of effluents from wastewater treatment plants.

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